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ORIGINAL RESEARCH

Preliminary evaluation of treatment efficacy of umbilical cord blood-derived mesenchymal stem cell-differentiated cardiac progenitor cells in a myocardial injury mouse model

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Abstract— Recently, stem cell therapy has been investigated as a strategy to prevent or reverse damage to heart tissue. Although the results of cell transplantation in animal models and patients with myocardial ischemia are promising, the selection of the appropriate cell type remains an issue that requires consideration. In this study, we aimed to evaluate the effect of cardiac progenitor cell transplantation in a mouse model of myocardial ischemia. The cardiac progenitor cells used for transplantation were differentiated from umbilical cord blood mesenchymal stem cells. Animal models injected with phosphate-buffered saline (PBS) and healthy mice were used as controls. Cell grafting was assessed by changes in blood pressure and histological evaluation. After 14 days of transplantation, the results demonstrated that the blood pressure of transplanted mice was stable, similar to healthy mice, whereas it fluctuated in PBS-injected mice. Histological analysis showed that heart tissue had regenerated in transplanted mice, but remained damaged in PBS-injected mice. Furthermore, trichrome staining revealed that the transplanted mice did not generate significant amount of scar tissue compared with PBS-injected control mice. In addition, the cardiac progenitor cells managed to survive and integrate with local cells in cell-injected heart tissue 14 days after transplantation. Most importantly, the transplanted cells did not exhibit tumorigenesis. In conclusion, cardiac progenitor cell transplantation produced a positive effect in a mouse model of myocardial ischemia.

Key words: cardiac progenitor cells; mouse model; myocardial ischemia; stem cell; transplantation.

INTRODUCTION

%&'($)*'$ +,-)$ *,./01'2)3(45$ -16'2)6/61$,7&,21'-$ 62$ 8976/462:$(6-;$/,1)9(-5$7(3:$)(',)8'2)5$,27$-3(:61,.$ 62)'(&'2)692-$*,&'$-6:26/61,2).4$68+(9&'7$)*'$<3,.6)4$ 9/$.6/'$,27$.6/'-+,2$9/$+,)6'2)-$=6)*$7,8,:'$)9$)*'$1,(0 76,1$83-1.'$/9..9=62:$8491,(76,.$6-1*'86,>$?9='&'(5$ 13((('2)$)(',)8'2)$-)(,)':6'-$/,6.$)9$('&'(-'$-1,($/9(8,0)692$9($('+,6($)*'$62@3('7$&'2)(61.'$=,..$,/)'($8491,(0 76,.$62/,(1)692$AB CD5$=*61*$'&'2)3,..4$.',7-$)9$&'20)(613.,($74-/321)692$,27$,((*4)*86,>$E3((('2).45$-)'8$ 1'..$)*'(,+4$6-$192-67'('7$,-$,$-)(,)':4$=6)*$:(',)$+90)'2)6,.$)9$+('&'2)$9($('&'(-'$1,(76,1$83-1.'$7,8,:'$,27$+(989)'$)6--3'$(':'2'(,)692>$

F*'$+('1.6261,.$897'.$9/$6-1*'861$*',()$76-',-'$*,-$ (,6-'7$,=,('2'--$,G93)$)*'$-,/')4$,27$'//616'214$9/$ -)'8$1'..$)*'(,+4$AH3443 86$')$,.>5$IJKKD>$L'-+6)'$)*'$ +(986-62:$('-3.)-$9/$1'..$)(,2-+.,2),)692$62$,268,.$ 897'.-$,27$+,)6'2)-$=6)*$6-1*'861$1,(769849+,)*45$6)$ ('8,62-$)9$G'$7')'(862'7$=*61*$1'..$)4+'$6-$)*'$89-)$,++(9+(6,)'$/9($)*6-$)*'(,+4$E3((('2)$1'..$)*'(,+6'-$/9($ *',()$76-',-'$*,&'$-*9=2$('8,(;,G.'$7'&'.9+8'2)-$ 198+,('7$=6)*$)*'$/6(-)$1'..$)(,2-+.,2),)692$+'(/9(8'7$ /9($*',()$7,8,:'$89('$)*,2IJ4',(-$,:9$AB,('..6$')$,.>5$KMMID>$C26)6,..45$-16'2)6-)-$/913-'7$92$3-62:$-)'8$ 1'..-$,27$-;'.'),.$83-1.'$-,)'..6)'$1'..-$G'1,3-'$)*'4$ ='('$','-6.4$67'2)6/6'75$,27$'N*6G6)'7$-)(92:$+(9.6/'(,0)692$(,)'-$,27$*6:*$6-1*'86,0=6)*-),2762:$,G6.6)4$

MATERIALS AND METHODS

G974$='6:*)>$a/)'($.'/)$)*9(,19)984$$.6:,)692$9/$)*'$.'/)$,2)'(69($7'-1'2762:$,()'(4$A`aLD$=,-$+'(/9(8'7$,)$ )*'$76-),.$K_"$9/$)*'$19(92,(4$,()'(4$/(98$)*'$,9(),$)9$ )*'$*',()$,+'N$AL':,G(6'.'$')$,.>5$IJJ!D>$a/)'($"$8625$ EXE-$='('$62/3-'7$62)9$)*'$,(',$9/$62/,(1)6925$,)$,$ 7'2-6)4$KJP$1'..-$+'($"J$µ`$/9($',1*$893-'>$F*'25$)*'$ 1*'-)$=,-$1.9-'75$,27$)*'$83-1.'$.,4'($=,-$-'=2$)6:*)$,27$,++.6'7$=6)*$,2)6-'+)61>$F*'$893-'$=,-$+.,1'7$ 1,('/3..4$62)9$,$1.',2$1,-'>$c9(8,.$861'$=('($3-'7$,-$ +9-6)6&'$192)(9.-5$=*6.'$`aL$.6:,)692$+.3-$XUR$62@'10)692$=,-$3-'7$,-$,$2':,)6&'$192)(9.>$

F(,2-+.,2),)692$'//61,14$=,-$,--'--'7$,119(762:$)9$ 1(6)'(6,5$621.3762:$G.997$+('--3('$/.31)3,)6925$,27$)*'$ -3(&6&,.5$86:(,)6925$,27$62/.3'21'$9/$EXE-$92$)*'$:(,/)'7$*',()$)6--3'>$

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B61'$G.997$+('--3('$1*,2:'-$=('($8926)9('7$3-62:$,$ #V#JJ$U.997$X('--3('$Q'19(7'($A]:9$U,-6.'R(.5$C),.4D5$ KgI$='';-G'/9('$,27$,/)'($1'..$)(,2-+.,2),)692>$

F9$/,16.6),)'$=6)*$-)('--5$861'$=('$)(,62'7$)9$G'$/,86.0 6,($=6)*$),6.013//$G.997$+('--3('$8',-3('8'2)->$F*'$,268,.-$=('$,2'-)*')6S'7$G4$+.,162:$)*'8$62$,2$,2'-0)*'-6,$1.9-'7$1*,8G'($$=*61*$+(9&67'7$192)62393-$ -3++.4$9/$#e$+3('$9N4:'2$,)$,$(,)'$K$`_862$9/$6-9/.30 (,2'5$32)6.$198,$=,-$62731'7>$F*'$893-'$),6.$=,-$620 -'()'7$62)9$)*'$G.997$+('--3('$8',-3(62:$(62:$=6)*$ -6:2,.$-'2-9(>$R4-)9.61$ARhRD$,27$76,-)9.61$ALCaD$G.997$ +('--3('$9/$)*'$,268,.-$=('$8',-3('7$,27$('19(7'7$ /9..9=62:$)*'$8,23/,1)3('(d-$62-)(31)692-$A#VPJJ$ U.997$X('--3('$Q'19(7'(D>$B',2$,()'(6,.$+('--3('$ AB aXD$=,-$1,.13.,)'7$G4$)*'$/9..9=62:$/9(83.,^$

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F*'$,268,.$*',()$=,-$=,-*'7$=6)*$XUR$,27$/6N'7$62$!e$+,(,/9(8,.7'*47'$9&'(26:*)>$a/)'($-31(9-'$1(490 +(9)'1)6925$)*'$)6--3'$=,-$893.7'7$=6)*$9+)6838$13)0)62:$)'8+'(,)3('$198+9327$,27$1(49-'1)692'7$62)9$ KJ0µ80)*61;$-.61'->$F*'$9G),62'7$-.61'-$=('$)*'2$)(',)'7$=6)*$*'8,)9N4.62$,27$'9-62$A?nTD$-),625$ F(61*(98'$-),625$,27$688329*6-)91*'86-)(4$$

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?nT$-),6262:$=,-$+'(/9(8'7$)9$'&,.3,)'$)6--3'$,27$ 1'..$-)(31)3('->$R.61'-$=('$688'(-'7$62$,$:(,7'7$-'0 (6'-$9/$,.19*9.$-9.3)692-$,-$/9..9=-^$KJJe$/9($I8625 MJe$/9($I8625ZJe$/9($K8625#Je$/9($K8625,27$7930 G.'076-)6..'7$=,)'($/9($I$862>$F*'$-3((932762:$)6--3'$,(',$=,-$7(6'7$=6)*$,G-9(G'2)$+,+'(5$/9..9=-'7$G4$,2$

,776)692$9/$,$7(9+$9/$*'8,)9N4.62$-),62$92)9$)*'$)6--3'$ -.61'>$a/)'($I8625)*'$-.61'$=,-$=,-*'7$=6)*$76-)6..'7$ =,)'(>$F=9$7(9+-$9/$'9-62$-),62$=('($,77'7$)9$)*'$)6-0 -3'$-.61'$/9($"J$-$,27$)*'$-+'168'2$=,-$-311'--6&'.4$ '8G'77'7$,:,62$=6)*^$#Je$,.19*9.$/9($K8625ZJe$,.0 19*9.$/9(K8625MJe,.19*9.$/9($K8625,27$KJJe$,.190 *9.$/9($K$862>$C88'(-692$96.$=,-$76-+'2-'7$92)9$)*'$ -.61'$/9..9='7$G4$.,8'..'$/6N,)692>$

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B,--92d-$F(61*(98'74'$6-$19889 2.4$3-'7$)9$76-)620 :36-*$19..,:'2$62$+,)*9.9:61,.$1,-'->$F*('$74'-$,('$ 3-'7$)9$67'2)6/4$19..,:'25$/6G(625$,27$'(4)*(914)'->$F6-0 -3'$=,-$626)6,..4$-),62'7$=6)*$U6'G(61*$R1,(.')$,16761$ 74'$A.6:*)$('7D5$=*61*$*,-$,$G62762:$+('/'('21'$)9$,16761$198+92'2)-62)*'$)6--3'>$Y*'2$)*'$)6--3'$=,-$)(',)'7$=6)*$+*9-+*989.4G761$,1675$)*'$('7$19.9($=,-$ 76-+.,1'7$/(98$)*'$19..,:'2$198+92'2)>$C2$,776)6925$ +*9-+*989.4G761$,167$,-9$:'2'(,)'7$.62;-$G')=''2$ 19..,:'2$,27$.6:*)$:(''2$89.'13.'-$)*,)$),62)'7$)*'$ 19..,:'2$:(''2>$

F6--3'$-+'168'2-$=('$688'(-'7$62$,$:(,7'7$-'(6'-$9/$,.19*9.$-9.3)692-$,-$/9..9=-^$KJJe$/9($K8625MJe$/9(K 8625$,27$VJe$/9($K$862>$R,8+.'-$=('$=,-*'7$:'2).4$)=61'$=6)*$76-)6..'7$=,)'(5$/9..9='7$G4$688'(-692$62$ U93620192),62$62:$-9.3)692$9&'(26:*)$,)$#P$oE>$R,8+.'-$ =('$(62-'7$=6)*$=,)'($)9$('89&'$)(,1'-$9/$+6(61$,167$ A4'..9=D$,27$-9,;'7$62$Y'6:'()d-$6(92$*'8,)9N4.62$ -9.3)692$/9($KJ$862>$R,8+.'-$=('($:'2).4$=,-*'7$1920)62393-.4$=6)*$76-)6..'7$=,)'($/9($"J$->$c'N)5$)*'4$=('$ '8G'77'7$62$U6'G(61*$-1,(.')$-9.3)692$/9($K$862>$F*'$ -.61'-$=('($=,-*'7$<361;.4$,:,62$=6)*$76-)6..'7$=,)'($,27$688'(-'7$62$#e$+*9-+*989.4G761$,167$-9.3)692$ /9($"J$862>$F*'25$-,8+.'-$=('$)(,2-/'(('7$,27$ G,)*'7$62$)*'$.6:*)$:(''2$-9.3)692$/9(KJ862>$F*'$ -,8+.'-$=('($(62-'7$<361;.4$=6)*$76-)6..'7$=,)'($,27$ 688'(-'7$62$J>#e$,1')61$,167$-9.3)692$/9(I862>$F*'$ -,8+.'-$=('$688'(-'7$62$,$-'(6'-$9/$:(,7'7$,.19*9.$ -9.3)692-$,-$/9..9=-^$M#e$/9(K862kI5$/9..9='7$G4$ KJJe$/9($K862k$I>$C88'(-692$96.$=,-$7(9++'7$92)9$)*'$-.61'5$/9..9='7$G4$.,8'..'$/6N,)692>$F(61*(98'0 -),62'7$-'1)692-$=('$67'2)6/6'7$,27$19..,:'2$-)(310)3('-$=('($'&,.3,)'7$G4$C8,:'p$-9/)=,('>$

I$$>A1?F'1?"&$04'6F%4'#0A0AC%%

E'..-$:(9=2$92$19&'(-.6+-$AR,2),$E(3S$U69)'1*29.9:45$ L,..,-5$Fq5$]RaD$=('$+('+,('7$/9($688329140 1*'86-)(4$,-$/9..9=-^$1'..-$=('$/6N'7$62$!e$+,(,/9(0 8,.7'*47'$-9.3)692$/9($K#$8620$+'(8',G6.6S'7$=6)*$ J>Ke$F(6)92$q0KJJ0$=,-*'7$*('')68'-62XUR0$G.91;'7$ =6)*$URa0$,27$6213G,)'7$=6)*$,$+(68,(4$*38,2$,2)60

G974$, .+* , 0 , 1)6262$AaG1 , 8$K^!JJD$9($*3 8 , 2$F(9+92 62$ F$AaG1 , 8$K^!JJD$9&'(26 : *)$,)$!$oE>$a/)'($= , -*62 :5$)*'$ - , 8 +.'-$ ='('$)(' ,)'7$ =6)*$: 9 ,)$, 2)60(, GG6)$C : \$-'1920 7 , (4$, 2)6G976'-$ AaG1 , 8$ K^!JJD$, 27$?9'1*-)$ """!I$ AR6 : 8 , $K^!JJD$ /9($!#$ 8 62$,)$ (99 8 $)' 8 +'(,)3('>$F*'$ - , 8 +.'-$ ='('$(62-'7$)*(''$)6 8 '-$ =6)*$XUR5$ 8 932)'75$, 27$ 9G-'(&'7$ 327'($, $ /.39('-1'2)$ 8 61(9-19+'$AW'6--$ aN69&'()D>$

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F6--3'$-'1)692-$ ='('$/6N'7$, 27$-) , 62'7$ =6)*$?9'1*-)$ """!I$AR6 : 8 , 5$K^!JJD$, 27$, 2)6G976'-$-+'16/61$/9($*3 8 , 2$ +(9)'62-$ 9/$ 62)'('-)5$ F(9+92 62$ F$AaG1 , 8 5$ K^IJJD$, 27$, .+* , 0 , 1)6262$AaG1 , 8 5$K^IJJD5$, 119(762 : $)9$)*'$ 8 , 230 / , 1)3('(d-$ +(9)919.>$ R.67'-$ 192) , 6262 : $)6--3'$ -'1)692-$ ='('$= , -*'7$G(6'/.4$/9($ Ik#$ 8 62$62$XUR>$F6--3'$-'10)692-$ ='('$G.91 ; '7$ =6)*$# e$URa$,)$(99 8 $)' 8 +'(,)3('$ /9($"J$ 8 62$, 27$6213G ,)'7$ =6)*$+(6 8 , (4$, 2)6G976'-$,)$ (99 8 $)' 8 +'(,)3('$/9(PJ 8 62>$ a/)'($ = , -*62 :5$-.67'-$ ='('$)*'2$)(' ,)'7$ =6)*$-'1927 , (4$, 2)6G976'-$,)$(99 8 $)' 8 +'(,)3('$/9(PJ 8 62$62$)*'$, G-'21'$9/$.6 : *)$)9$, &967$ 9+)61 , .$G.' , 1*62 : >$F*'$- , 8 +.'-$ ='('$= , -*'7$!k#$ 8 62$ 62$ XUR5$ 19 ,)'7$ =6)*$ +9.4&624.$, .19*9.$ -9.3)6925$, 27$ 19&'('7$G4$. , 8 '..'>$F*'6($6 8 , : '-$ ='('$9G-'(&'7$, 27$ ('19(7'7$G4$/.39('-1'21'$ 8 61(9-19+4>$

3&E&64&%'6#A4?60B'01A<B1(F $ &6#4&%?"#0A%6&#?'01A%%

U'/9('$)(, 2-+. , 2) ,)6925$ EXE-$ ='('$)'-)'7$ /9($)*'$ 'N+('--692$9/$*3 8 , 2$R9NI$, 27$%1)!$: '2'-5$ =*61*$, ('$ 3-'7$)9$1* , (, 1)'(6S'$)3 8 9($/9(8 ,)6925$3-62 : $('&'(-'$)(, 2-1(6+)6920+9.4 8 '(, -'$ 1* , 62$ (' , 1)692$ AQF0XEQD>$?3 8 , 2$: .41'(, .7'*47'$"0+*9-+* ,)'$7'*47(9 : '2 , -'$ A*\ aXL ?D$= , -$3-'7$, -$, 2$62)'(2 , .$192)(9.>$

a)$ K!$ 7 , 4-$ +9-)0)(, 2-+. , 2) ,)6925$)9) , .$ Q c a$ /(9 8 $ *' , ()-$9/$192)(9.$, 26 8 , .-$9($)'-)$, 26 8 , .-$ = , -$19..'1)'7$)9$'N , 8 62'$)*'$)3 8 9($/9(8 ,)692$1 , + , 16)4$ 9/$ 62/3-'7$ 1'..-62&6&9>$

F9) , .$ Q c a$ = , -$ 'N)(, 1)'7$ /(9 8 $ EXE-$ +('+ , ('7$ /9($)(, 2-+. , 2) ,)6925$*3 8 , 2$B E f Z$G(' , -)$1 , 21'($1'..-5$, 27$ *' , ()$)6--3'$- , 8 +.'-$K!$7 , 4-$+9-)0)(, 2-+. , 2) ,)692$3-0 62 : $T , -4$U.3'$Q' , : '2)$AC2)(92D5$/9..9=62 : $)*'$ 8 , 230 / , 1)3('(d-$ 62-)(31)692->$ 1L c a$ = , -$ -42)*'-6S'7$ /(9 8 $ Q c a$ 3-62 : $)*'$R3+'(-1(6+)CC Q'&'(-'$ F(, 2-1(6+) , -'$ AC2&6)(9 : '2D5$, 119(762 : $)9$)*'$ 8 , 23/ , 1)3('(d-$ +(91'0 73('>$ c 'N)5XEQ = , -$ +'(/9(8 '7$3-62 : $F , <$L c a$X90 .4 8 '(, -'$AF , ; , (, 5$R*6 : , 5$p , + , 2D>$F*'$ +(6 8 '(-$ 9/$)*'$ 'N , 8 62'7$: '2'-$, ('$.6-)'7$62$5#J(&%->$

\ '.$ '.'1)(9+*9('-6-$ = , -$+'(/9(8 '7$92$QF0XEQ$+(970 31)-92I e$, : , (9-'$: '.-5$,)KJJb5$/9(PJ 8 62>aKJJ0G+$ L c a$. , 77'($= , -$ 3-'7$ /9($, ..$ +(9731)->$F*'$ ('-3.)-$ ='('$, 2 , .4S'7$G4$ '.'1)(9+*9('-6-$3-62 : $)*'$\ '.$L 91$CF$ -4-)' 8 $A] bXD>$

Table 1. Primers of the examined genes

Gene	Primer	Ta	Cycles	Product size (bp)
hSox2	F: CAACGGCAGCTACAGCA R: GGAGTGGGAGGAAGAGGT	60°C	35	283 bp
hOct4	F: TGGGGGTTCTATTTGGGAAGG R: GTTCGCTTTCTCTTTCGGGC	60°C	35	193 bp
hGAPDH	F: GTCAACGGATTTGGTCGTATTG R: CATGGGTGGAATCATATTGGAA	60°C	35	139 bp
mGAPDH	F: GCATGGACTGTGGTCATGAG R:CCATCACCATCTTCCAGGAG	60°C	35	322 bp

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K'#'04'0?#(%L A#(F404%

L ,) , $ ='('$ -) ,)6-)61 , ..4$ 62)'(+(')'7$ 3-62 : \(, +*X , 7$ X(6- 8 $-9/) = , ('$&'(-692$P>$

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RESULTS

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]EU0BRE-$='('$62731'7$62$8'7638$192),6262:$#0 aS,>$F*'$62731'7$1'..-$='('$1*'1;'7$G4CEE-),6262:$ =6)*$,2)6G976'-$-+'16/61$/9($*38,2$1,(76,1$83-1.'$ 1'..-5$621.3762:$*38,2$,.+*,0,1)6262$,27$*38,2$)(90 +9262$F>$F*'$('-3.)-$62761,)'7$)*,)$,$+*'29)4+61$ 1*,2:'$9113(('7$62$)*'$62731'7$1'..->$%(6:62,..45$)*'$ +9+3.,)692$'N*6G6)'7$)*'$89(+*9.9:4$9/$BRE-5$=*61*$,('$8929231.',($,27$-+627.'$-*,+'70$)*'25$)*'4$)(,2-0 /9(8'7$62)9$83.)6231.',($1'..-5$=*61*$,('$-686.,($)9$)*9-'$9/$1,(76,1$83-1.'$1'..$.62',:'->$a776)692,..45$)*'4$,.-9$'N+('--'7$G9)*$-+'16/61$8,(;'(-$9/$1,(76,1$ 83-1.'$1'..-5$*38,2$,.+*,0,1)6262$,27$*38,2$)(90 +9262FAN0C)%-D>$

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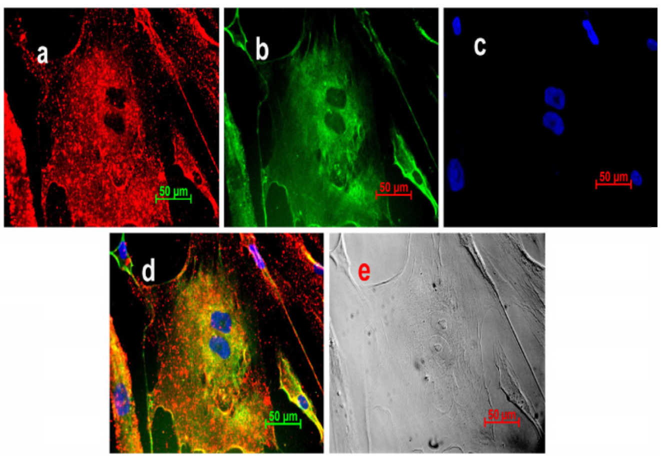


N0C>6&%-)%I$$>A1?F'1?"&$04'6F%6&4>('4%1@%0A2>?&2%?&((4>$ A,D$C2731'7$1'..-$='('$+9-6)6&'$/9($*38,2$,.+*,0,1)6262$0AGD 62731'7$1'..-$='('$+9-6)6&'$/9($*38,2$)(9+9262$F0$A1D$1'..$ 231.'6$='('$-),62'7$=6)*$?9'1*-)$"""!I0$A7D$9&'(.,4$68,:'$ 9/$68,:'-$,5G5$,27$10$A'D$62731'7$1'..$68,:'$=,-$1,+)3('7$ 327'($=*6)'$.6:*)>$R1,.'$G,(5$#J$μ8>%

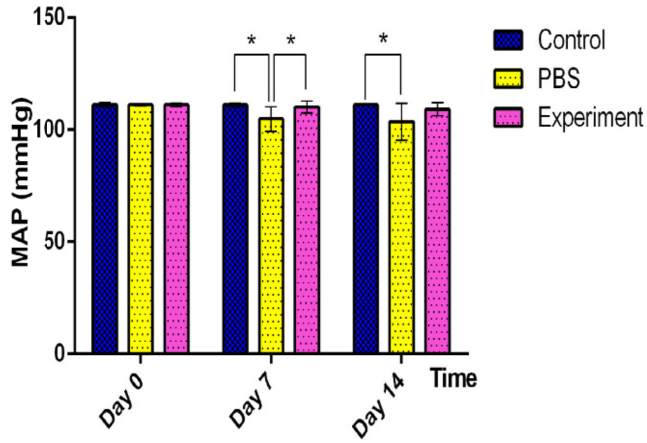
%

%

/(112%B6&44>6&%?"#AC&4%

F9$,--'--$)*'$'//'1)$9/$1'..$)(,2-+.,2),)692$62$)*'$893-'$ 897'.$9/$8491,(76,.$6-1*'86,5$1*,2:'-62G.997$+('-0 -3('$G'/9('$,27$,/)'($)(,2-+.,2),)692$='('$('19(7'7$)*(93:*$,$7'761,)'7$-4-)'8$AN0C)%+D>$F*'$19..'1)'7$7,),$ ='('$+(91'--'7$3-62:$-),)6-)61,.$-9/)=,('>$

%



N0C>6&%+)%/(112%B6&44>6&%?"#AC&4)%U.997$+('--3('$-),G6.6S'7$ 62$29(8,.$*',.)*4$861'$,27$)'27'7$)9$7'1.62'$62$XUR0 62@'1)'7$861'>$L3(62:$)*'$-3(&'4$)*'$G.997$+('--3('$9/$)(,2-+.,2)'7$861'$=,-$7'1(',-'7$5$G3)$29)$-6:26/61,2).419$80 +,('7$=6)*$XUR062@'1)'7$861'>$Q'-3.)-$,('$8',2-t-),27,(7$ 7'&6,)692$0$R)37'2)d-$'0)'-)5$+r$J>J#^$ s$62761,)'-$-6:26/61,2)$ 76//'('21')%

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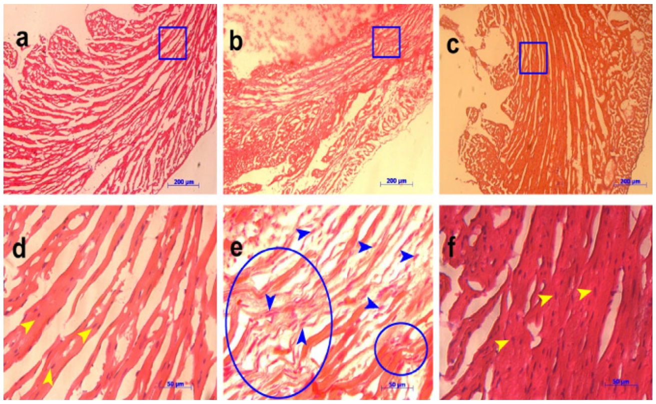
C2$29(8,.$861'5$)*'$BaX$=,-$,++(9N68,)'.4KKK 88?:$AF6'8,22$')$,.>5$IJJ"D>$?',.)*4$861'$A2$iKJD ='('$3-'7$,-$,$+9-6)6&'$192)(9.$=6)*$-),G.'$G.997$+('-0 -3('$ALJiKKK>K$88?:0$LZiKKK>J$88?:0$LK!i KKK>K$88?:D>$?9='&'(5BaX62$)*'$`aL$.6:,)692$,27$ XUR062@'1)'7$861'$AXUR$:(93+D$A2iKJD$)'27'7$)9$7'0 1(',-'$9&'($)*'$-3(&'4$+'(697$ALJiKKK>J$88?:0$LZi KJ!>V$88?:0$LK!iKJ">#$88?:D>$F*6-$('-3.)$-3:0 :'-)'7$)*,)$*',()$,1)6&6)4$=,-$7'1(',-'7$62$)*'$XUR$:(93+>$B',2=*6.'5$)*'BaX62$)*'$EXE0)(,2-+.,2)'7$:(93+$A2$iKJD=,-$7'1(',-'7$198+,('7$=6)*$)*'$1920)(9.$:(93+ALJi$KKK>J$88?:0LZi$KKJ>K$88?:0$LK!$ i$KJM>K$88?:D0$*9='&'(5$)*'$7'1(',-'$=,-$'&67'2).4$.'--$)*,2$)*,)$9/$)*'XUR:(93+>$$

$

=!=%'6#A4B(#A'#'01A%6&2>?&2%"&#6'%'044>&%2#$#C&4%%

F9$,--'--$)*'$'//'1)-$9/$:(,/)'7$EXE-62)*'$893-'$ 897'.5$,268,.-$='('$76&67'7$62)9$)*('$:(93+-$F*'$ 192)(9.$:(93+$192-6-)'7$9/$29(8,.$*',.)*4$861'0$)*'$ XUR$:(93+$192-6-)'7$9/$861'$('1'6&62:$`aL$.6:,)692$ ,27$XUR$62@'1)692$,27$)*'$EXE$:(93+$198+(6-'7$861'$ ('1'6&62:$`aL$.6:,)692$,27EXE)(,2-+.,2),)692>$



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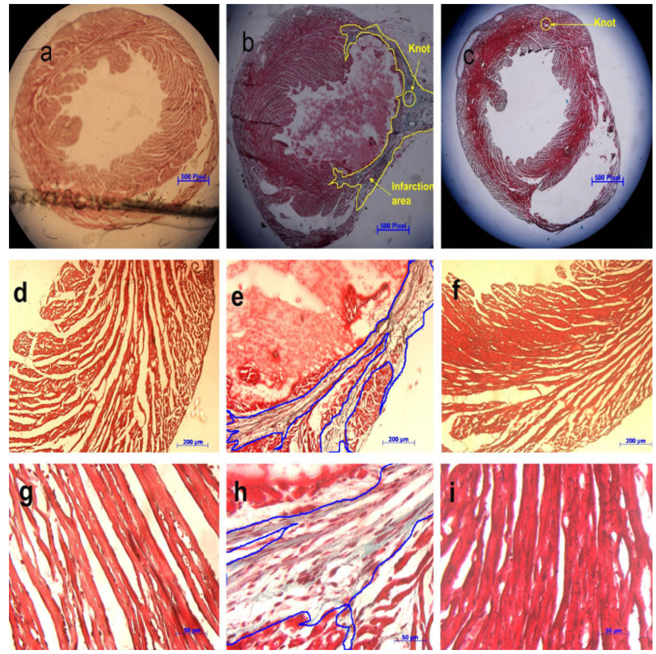
A,5$7D$*',()$)6--3'$9/$192)(9.$:(93+$=,-$*',.)*4$,27$
32G(9;'20$AG5$'D$*',()$)6--3'$9/$)*'XUR:(93+$=,-$
/(,1)3('75$(6++.'7$AG.3'$16(1.'7$,(',D5$,27$'N*6G6)'7$
;,(49.4)61$231.'6$AG.3'$,((9=D0$A15$/D$*',()$)6--3'$9/$)*'$
EXE$:(93+$=,-$29)$(6++.'7$,27$)*'$231.'6$='('$62),1)$
62$)*'$1'2)'($9/$)*'$1'..$A4'..9=$,((9=D>$

F*'$*',()$)6--3'$=,-$/6N'7$,27$1(490-'1)692'7$92)9$
-.67'-$AKJ0µ80)*61;$-.61'-D>$R.67'-$=('$)*'2$-),62'7$
=6)*$?nT$/9($)6--3'$7,8,:'$,--'--8'2)>$Q'-3.)-$
-*9='7$)*,)$*',()$)6--3'$62$)*'$192)(9.$:(93+$('0
8,62'7$62),1)$,27$'N*6G6)'7$29$-6:2$9/$62/.,88,)692>$
F*'$.'/)$&'2)(61.'$=,..$9/$)*'$192)(9.$:(93+$=,-$29(0
8,.5$=*6.'$6)$=,-$)*622'(62)*'XUR:(93+>$?',()$)6-0
-3'$9/$)*'XUR:(93+$'N*6G6)'7$89('$.9--$9/$1(9--0
-)(6,)692-5$=6)*$-6:26/61,2).4$-*(32;'2$G,27-$,27$230
1.',($;,(49.4-6-$62$*',()$1'..-$A:(''2$,((9=5$N0C)%;D>$
R98'$2'3)(9+*6.-$62/6.)(,)'7$)*'$*',()$83-1.'$,27$/60
G'(-$,++',('7$(6++.'7$62$)*'XUR:(93+$A,(',$16(1.'7$
62$G.3'*%N0C)%;D>$?9='&'(5$29$/(,1)3('$9($7,8,:'$9/$)*'$
',()$)6--3'$=,-$9G-'(&'7$92$)'$EXE$:(93+>$C2$,7760
)692$5$)*'$231.'3-$=,-$.91,)'7$62$)*'$1'2)'($9/$)*'$1'..$
A4'..9=$,((9=-5$N0C)%;D5$+,.'$+62;$+.,)'-$=('$,-9$9G0
-'(&'7$,27$)*'$.'/)$&'2)(61.'$=,..$=,-$29)$,-$)*62$,-$)*'
XUR$:(93+$AN0C)%;D>$

F9$,--'--$-1,($/9(8,)692$62$)*'$7,8,:'7$*',()$)6--3'$
,27$'//'1)-$9/$EXE-$92$)*'$+(91'--$861'$*',()-$=('$
19..'1)'7$/9($F(61*(98'$-),62>$Q'-3.)-$-*9='7$)*,)$
)*'$8491,(763$8$9/$)*'$192)(9.$:(93+$767$29)$'N+'(60
'21'$/6G(9-6-$AN0C)%:#*%:2*%:CD5$=*6.'$)*'XUR:(93+$
/9(8'7$G(9,7$G.3'$(':692-$9/$/6G(9-6-$=*'2$-),62'7$
=6)*$F(61*(98'$AN0C)%:J*%:&*%:"D>$c9$-6:2$9/$/6G(9-6-$
=,-$9G-'(&'7$62$)*'$8491,(763$8$9/$)*'$EXE$:(93+$
198+,('7$=6)*$)*'XUR:(93+$AN0C)%:?*%:@*%:0D>$



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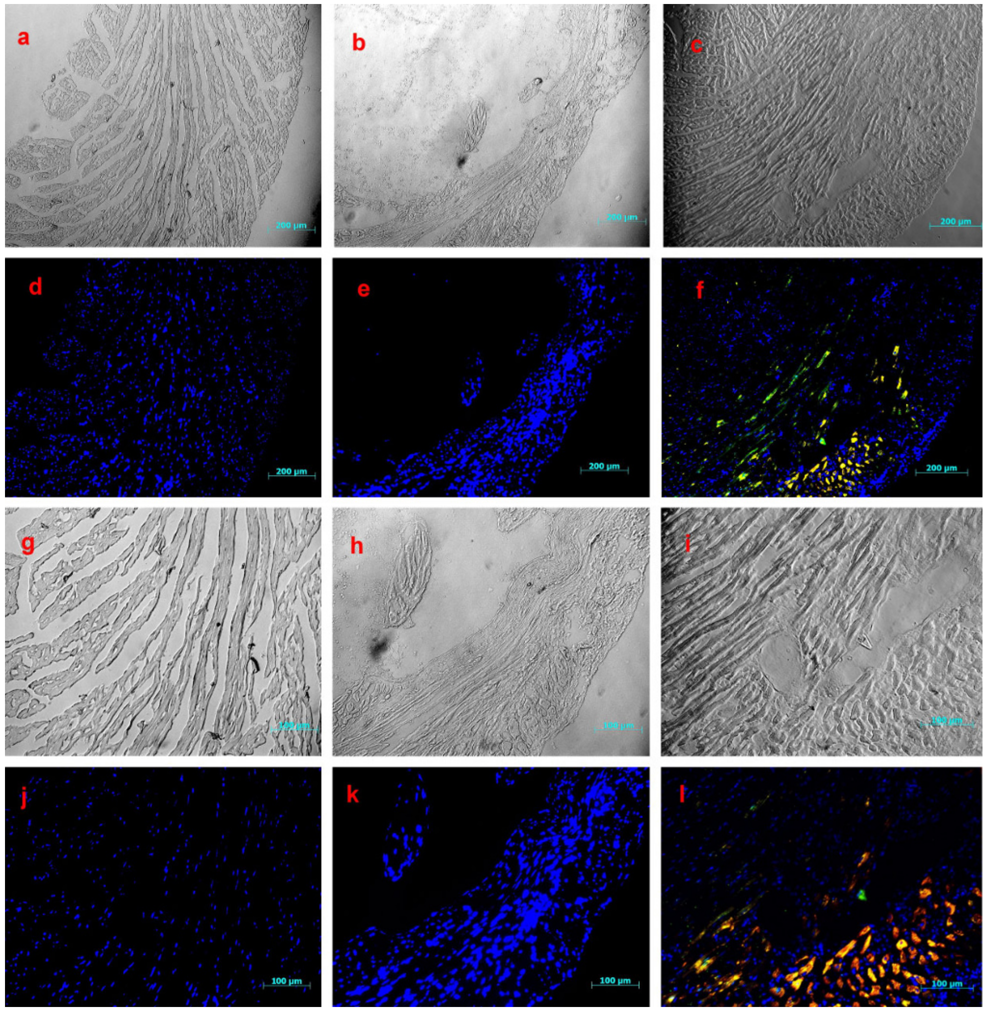
A,5$75$:D$?',()$)6--3'$9/$)*'$192)(9.$:(93+$'N*6G6)'7$29$-1,($
/9(8,)692$AG5$'5$*D$*',()$)6--3'$9/$)*'$XUR$:(93+$/9(8'7$,$
.,(:'$-1,($A15$/5$6D$*',()$)6--3'$9/$)*'EXE:(93+$,.-9$'N*6G0
6)'7$29$-1,($/9(8,)692$5$)$K!$7,4-$+9-)0$68+.,2),)692>%

!14'<'6#A4B(#A'#'01A%&E#(>#'01A%1@%0A@>4&2%=!=4%0A%
"&#6'%'044>&%

F9$62&'-)6:,)'$)*'$/,)'$9/$)*'$62/3-'7$EXE-$62$)*'$7,80
,:'7$*',()$)6--3'$9/$'N+'(68'2)$,268,.-$893-'$*',()$
)6--3'$-,8+.'-$=('$19..'1)'7$/(9S'25$,27$-'1)692'7>$
R.61'-$=('$)*'2$-),62'7$=6)*$,2)6G976'-$=*61*$=('$
-+'16/61$/9($*38,2$*',()$1'..-5$621.3762:$*38,2$,.+*,0
,1)62$,27$*38,2$)(9+9262$F>$

F*'$('-3.)-$9/$)6--3'$-),62:$62761,)'7$)*,)$*',()$)6-0
-3'-$9/$)*'$192)(9.$,27XUR:(93+-$=('$2':,)6&'$/9($
-),62:$=6)*$)*'-'$-+'16/61$,2)6G976'-$AN0C)%.#*%2*%C*%R*%
J*%&*%"*%QD>$B',2=*6.'5$-98'$,(',-$9/$EXE0
)(,2-+.,2)'7$*',()$)6--3'$'N*6G6)'7$+9-6)6&'$-),62:$
AN0C)%.?*%@*%0*%(D5$62761,)62:$)*,)$:(,/)'7$EXE-$-3(&6&'7$
,27$'N+('--'7$G9)*$*38,2$F(9+9262FA:(''2D$,27$
38,2$,.+*,0,1)62$A('7D>$F'-'$1'..-$29)$92.4$767$
29)$/9(8$-1,($)6--3'$G3)$)*'4$,.-9$+('&'2)'7$9($('0
731'7$)6--3'$7,8,:'$1,3-'7$G4$6-1*'86,>$F*'('/9('5$
)*'$.'/)$&'2)(613.,($=,..-$9/$EXE0)(,2-+.,2)'7$861'$
=('$('.,)6&'.4$)*61;$198+,('7$=6)*$29(8,.$861'>$C2$
192)(,-)5$861'$62$)*'$XUR$:(93+$'N*6G6)'7$)*62$&'20
)(613.,($=,..-$,27$-1,($/9(8,)692>$



N0C>6&%.)%D&#6'%'044>&%0$ $>A1"04'1?"&$04'6F%6&4>('4%1@%'"&%'"6&&%C61>B4>$A,5$75$:5$D$B491,(76,.$)6--3'$9/$29(8,.$861'$767$29)$-),62$=6)*$,24$9/$)*'$-+'16/61$,2)6G976'-$/9($*38,2$1,(76,1$83-1.'$,)$&,(693-$8,:26/61,)692-0$AG5$'5$*5$;D$*',()$)6--3'$62$)*'$XUR$:(93+$=,-$,.-9$2':,)6&'$/9($)*'-'$-+'16/61$,2)6G976'-0$A15$/5$65$.D$)(,2-+.,2)'7$EXE-$'N6-)'7$62$)*'$893-'$*',()$6--3'$,27$='('$+9-6)6&'$/9($G9)*$*38,2$,.+*,0,1)6262$,27$*38,2$)(9+9262$F>$f `$A f.93('-1'62D0$Y f$A=*6)'$/6'.7D>%

%

=!=4%   202%   A1'%   0A2>?&%   '>$160C&A&404%   B14'<
'6#A4B(#A'#'01A%

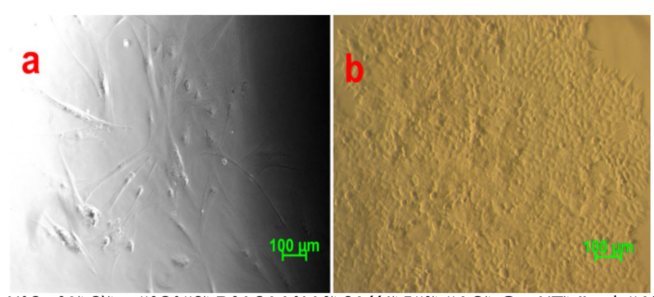
F9$)'-)$/9($)*'$)389(0/9(862:$,G6.6)4$9/$)*'$)(,2-0+.,2)'7$1'..-$+(69($)9$)(,2-+.,2),)692$$EXE-$='('$'&,.3,)'7$/9($*38,2$1,21'($:'2'$'N+('--692$$621.370 62:$*38,2$R9NI$A*R9NID$,27$*38,2$%1)!$A*%1)!D>$F*'$*38,2$G(',-)$1,21'($BEfZ$1'..$.62'$=,-$3-'7$,-$,$+9-6)6&'$192)(9.$AN0C)%SD$,27$76-)6..'7$=,)'($=,-$3-'7$,-$,$2':,)6&'$192)(9.>$F*'$('-3.)-$-*9='7$)*,)$)*'$EXE-$767$29)$'N+('--$*%1)!$,27$*R9NI$198+,('7$=6)*$BEfZ$1'..-$AN0C)%T#D>$

F9$,--'--$=*')*'(EXE-3(&6&,.$,27$)389(6:'2'-6-$62$893-'$ 1,(76,1$)6--3'$ +9-)0)(,2-+.,2),)692$$EXE0)(,2-+.,2)'7$)6--3'$=,-$19..'1)'7>$F9),.$Qc a$=,-$'N0)(,1)'7$/(98$*',()$)6--3'$/9($/3()*'($,2,.4-6-$9/$*R9NI$,27$*%1)!$'N+('--692>$Qc a$/(98$29(8,.$893-'$*',()$=,-$3-'7$/9($198+,(6-92>$BEfZ$1'..-$,27$76-)6..'7

=,)'($192)(9.-$=('$)(',)'7$=6)*$)*'$-,8'$+(91'73('>$F*'$('-3.)-$-*9='7$)*,)$)(,2-+.,2)'7$EXE-$'N6-)'7$62$:(,/)'7$)6--3'-$,27$)*'$)389($:'2'-$*R9NI$,27$*%1)!$='('$29)$'N+('--'7$AN0C)%TJD>$

$

$



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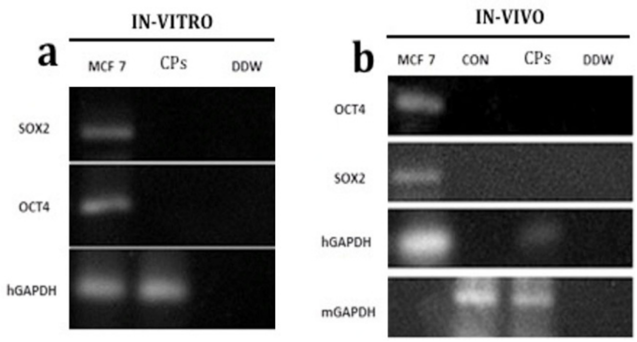

N0C>6&%T)%=#620#?%B61C&A0'16%?&((4%7=!=48%202%A1'%&GB6&44% "K1G+%#A2%"U?':)%A,D$EXE-$767$29)$'N+('--$*%1)!$,27$*R9NI$ =*'2$13.)3('7$!"#$!',%0$AGD$EXE-$'N6-)'7$62$)(,2-+.,2)'7$)6-0 -3'$,27$767$29)$'N+('--$*R9NI$,27$*%1)!$K!$7,4-$+9-)0)(,2-+.,2),)692!"#$!$%$

DISCUSSION

C2$)*6-$-)374$$='$'&,.3,)'7$)*'$+9)'2)6,.$9/$EXE-$)9$ ('+,6($7,8,:'7$8491,(76,.$)6--3'>$EXE$)(,2-+.,2),0)692$8626 86S'7$G.997$+('--3('$/.31)3,)692$62$)(,2-0 +.,2)'7$861'$-686.,($)9$)*'$192)(9.$:(93+0$,27$=,-$ 89('$-),G.'$198+,('7$=6)*$)*'XUR:(93+>$F*'$'20 :(,/)'7$EXE-$-3(&6&'7$,27$62)':(,)'7$=6)*$)*'$*9-)$ 1,(76,1$)6--3'5$68+(9&'7$)6--3'$-)(31)3('5$,27$+('0 &'2)'7$)*'$+(9:('--692$9/$/6G(9-6-62)*'$7,8,:'7$ *',()$83-1.'$K!$7,4-$,/)'($)(,2-+.,2),)692>$EXE$)(,2-0 +.,2),)692$767$29)$62731'$)'(,)98,$/9(8,)692$,27$)*'$ 'N+('--692$9/$1,21'($:'2'-5$621.3762:$*%1)!$,27$ *R9NI5$62$EXE0)(,2-+.,2)'7$861'>$C2$9)*'($-)376'-5$)*'$)(,2-+.,2),)692$9/$32 76//'('2)6,)'7$TRE-$62)9$(,)$8970 '.-$9/$BC$)'27'7$)9$:'2'(,)'$)'(,)98,-Ac3--G,38$')$,.>5$IJJZD>$C2$93($-)374$$)*'$)(,2-+.,2),)692$9/$EXE-$ 7'(6&'7$/(98$]EU0BRE-$767$29)$/9(8$)'(,)98,-62)(,2-+.,2)'7$)6--3'$7N0C)%S*%N0C)%T8>$F*6-$76//'('21'$8,4$ G'$,$('-3.)$9/$)*'$62731)692$9/$76//'('2)$1*,2:'-62)*'$:'2'$'N+('--692$9/$EXE-$,27$TRE-5$=*'('G4$EXE-767 29)$327'(:9$9219:'261$76//'('2)6,)692>$F*6-$('-3.)$=,-$ -686.,($)9$)*'$+('&693-$('-3.)-$9/$?3G'($&'#()*5$=*'('$ *38,2$TRE07'(6&'7$1,(7698 4914)'-$A*TRE0EB-D$ ='('$62/3-'7$62)9$,$893-'$897'.$9/BCA?3G'($')$,.>5$ IJJZD>$F*')$)(,2-+.,2),)692$9/$)*'-'$1'..-$,/)'(BC767$ 29)$.',7$)9$)*'$/9(8,)692$9/$)'(,)98,->$?9='&'(5$)*'$ '2:(,/)'7$1,(7698 4914)'-$,.-9$/,6.'7$)9$-3(&6&'5$+(90 .6/'(,)'5$9($62)':(,)'$=6)*$*9-)$1,(76,1$)6--3'$A?3G'($')$,.>5$IJJZ0$[.3:$')$,.>5$KMMPD>$C2$)*6-$-)374$$)*'$)(,2-0 +.,2)'7$EXE-$-3(&6&'7$,27$62)':(,)'7$=6)*$)*'$*9-)$ 1'..-$AN0C)%.D5$=*61*193.7G'$('.,)'7$)9$76//'('21'-$62$ 1'..$-93(1'-5$8926)9(62:$)68'5$,27$1'..$76//'('2)6,)692$

+(91'73('->$?9='&'(5$*TRE0EB$)(,2-+.,2),)692$680 +(9&'7$7,8,:'7$*',()$/321)692$62$,$-686.,($8,22'($,-$ EXE$)(,2-+.,2),)692>$U'1,3-'$1,(7698 4914)'$.9--$ +.,4-$,$-6:26/61,2)$(9.'$62$)*'$7'&'.9+8'2)$9/$*',()$ /,6.3('5$1,(7698 49:'2'-6-$6-$)*3-$,2$68+9(),2)$+(91'--$ 62$)*'$(':'2'(,)692$9/$*',()$)6--3'$A`,/.,88'$,27$ B3((4$5$IJJ#5$IJKKD>$C2)'('-)62:.4$$)*'$('-3.)-$9/EXE)(,2-+.,2),)692$,/)'($K!$7,4-$-*9='7$)*,)$)*'$.'/)$&'20)(613.,($=,..$9/$)*'$EXE062/3-'7$:(93+$=,-$)*61;'($ 198+,('7$=6)*$)*'XUR:(93+$Af6:3('$"D>$F(61*(98'$ -),6262:$('-3.)-$,.-9$192/6(8'7$)*'$,G-'21'$9/$-1,($ /9(8,)692$/(98$)*'$*',()$)6--3'$9/$)*'EXE:(93+$ Af6:3('$!D5$=*6.'$,$.,(:'$-1,($=,-$9G-'(&'7$62$)*'$*',()$)6--3'$9/$)*'XUR:(93+$Af6:3('$!D>$F*6-$('-3.)$-3:0 :'-)'7$)*,)$:(,/)'7$EXE-$68+(9&'7$1,(76,1$/321)692$ 7')'(69(,)692$62$7,8,:'7$*',()$)6--3'>$E3(('2).4$$)*'('$,('$-'&'(,.$)*'9(6'-92*9=$1'..$)(,2-+.,2),)692$680 +(9&'-$8491,(76,.$+'(/3-692$,27$1,(76,1$/321)692>$ F*'$)(,2-+.,2)'7$1'..-$,('$1,+,G.'$9/$76//'('2)6,)62:$ 62)9$8491,(76,.$1'..-$,/)'($62/3-692$,27$'&'2)3,..4$ 68+(9&'$1,(76,1$/321)692>$C2$,776)692$)*'$)(,2-0 +.,2)'7$1'..-$8,4$+9--'--$)*'$,G6.6)4$)9$('.','$ +,(,1(62'$/,1)9(-5$-31*$,-$&,-13.,($'279)*'.6,.$:(9=)*$ /,1)9(5$62-3.620.6;'$:(9=)*$/,1)9(5$*'+,)914)'$:(9=)*$ /,1)9(5$,27$/6G(9G.,-)$:(9=)*$/,1)9(5$=*61*$,1)6&,)'$ '279:'293-$1,(76,1$+(9:'26)9(-$)9$+(9)'1)$,27$('+,6($ .9-)$*',()$83-1.'>$C2$)*6-$-)374$6)$=,-$7'892-)(,)'7$)*,)$)*'$7'.6&'('7$EXE-$8,2,:'7$)9$-3(&6&'62)*'$:(,/)'7$)6--3'$,27$767$29)$)(,2-/9(8$62)9$83-1.'$/60 G'(-5$G3)$8,62),62'7$)*'$/321)692-$9/$1,(76,1$1'..-$AN0C)% :*%N0C)%.D>$C2$192)(,1)5$)*'$*',()$)6--3'$9/$)*'$XUR$:(93+$ =,-$-'&'('.4$7,8,:'7$AN0C)%:*%N0C)%.D>$F*6-$('-3.)$=,-$ -686.,($)9$+('&693-$-)376'-$,G93)$)*'$,G6.6)4$9/$1'..-$)9$ 62)':(,)'$=6)*$*9-)$1,(76,1$)6--3'$G9)*$!"#$!',%$,27$!"# $!$%A['*,)$')$,.>5$IJJ!0$q3'$')$,.>5$IJJ#D>$F*6-$'//'1)$=,-$ 67'2)61,.$)9$)*'$+9-6)6&'$'//'1)$9/$8'2-)(3,.$G.9970 7'(6&'7$BRE$AB BED$)(,2-+.,2),)692I='';-$,/)'($ `aL$.6:,)692$A?67,$')$,.>5$IJJVD0$9($-686.,($)9$)*'$('0 -3.)-$9/$+9(162'$6XRE$)(,2-+.,2),)692$62$,$BC$897'.$A`6$ ')$,.>5$IJK"D>$F*'$-686.,(6)4$G')=''2$)*'$:(,/)'7$B BE-$,27$6XRE-$=,-$)*,)$G9)*$'N*6G6)'7$)*'$,G6.6)4$)9$76//'(0 '2)6,)'$62)9$*',()$1'..-$,/)'($)(,2-+.,2),)692$,27$76-0 +.,4'7$+9-6)6&'$'//'1)-$62$1.6261,.$)(6,.->$E92&'(-'.4$ 1,(76,1$BRE-$/9(8'7$,$-1,($,/)'($)(,2-+.,2),)692$ AE,(.-92$')$,.>5$IJKK0$R6.&,$')$,.>5$IJK!D5$*TRE-$/9(8'7$)'(,)98,-$,/)'($)(,2-+.,2),)692$A?'2)S'$')$,.>5$IJJMD5$,27$UB0BRE-$*,7$29$62/.3'2)6,.$'//'1)-92)*'$:(,/)'7$ *',()$)6--3'$,/)'($62/,(1)692$A\(,3--$')$,.>5$IJJZ0$?93$')$,.>5$IJJZD>$B',2=*6.'5$)*'-'$1'..-$)4+'-$,1;$)*'$,G6.6)4$)9$76//'('2)6,)'$62)9$*',()$83-1.'$1'..-$,/)'($)(,2-+.,20),)692$=*'(',-$:(,/)'7$EXE-$='('$+9-6)6&'$/9($-+'16/61$

,2)6G976'-$9/$ 8491,(76,.$1'..-$G'/9('$,27$,/)'($)(,2-0 +.,2),)692$$=*61*$86:*)$'N+.,62$=*4$EXE$)(,2-+.,20),)692$=,-$-*9=2$)9$*,&'$,$/,&9(,G.'$'//'1)62,268 ,.$ 897'.-$198+,('7$=6)*$9)*'($1'..$)4+'->$a.)*93:*$)*'$ 626)6,.$('-3.)-$='('$'2193(,:62:5$,-$='..$,-$-*9=62:$ -6:26/61,2)$+9)'2)6,.$/9(EXE,++.61,)692$62$,268 ,.$ 897'.-$9/$7,8,:'7$*',()$)6--3'$1,3-'7$G4$6-1*'86,5$ /3()*'($-)376'-$-*93.7G'192731)'7$3-62:$,.)'(2,)6&'$ 8')*97-5$-31*$,-$'.'1)(91,(769:(,+*45$9($G4$621(',-0 62:$)*'$'N+'(68'2),.$'&,.3,)692$)68'.62'$)9$+(9731'$ -)(92:'($('-3.)->$

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CONCLUSION

F*'EX)(,2-+.,2),)692$)*'(,+4$62$)*'$893-'$897'.$9/$ 7,8,:'7$ 8491,(76,.$)6--3'$1,3-'7$G4$6-1*'86,$6260)6,..4$-*9='7$+9-6)6&'$'//'1)-$/9($)*'$+('&'2)692$9/$ 8491,(76,.$62@3(4$,/)'($`aL$.6:,)692>$

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Acknowledgments

F*6-$=9(;$=,-$-3++9()'7$G4$)*'$b6')2,8$c,)692,.$]26&'(-6)4$$?9$E*6$B62*%b6')2,8$327'($:(,2)$ AUIJKK0KV0JZFLD>$$

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Ethical standard statements

]8G6.61,.$19(7$G.997$-,8+.'-$19..'1)'7$/(98$62/9(8'7$ 192-'2)$9/$)*'$89)*'($3-'7$)9$6-9.,)'$BRE->$F*'$19.0 .'1)692$=,-$+'(/9(8'7$62$,119(7,21'$=6)*$)*'$')*61,.$ -),27,(7-$9/$)*'$.91,.$')*61-$19886))''$b,2$?,2*$ \'2'(,.$?9-+6),.5$?9$E*6$B62*$16)4$b6')2,8>$a..$+(90 1'73('-$,27$8,26+3.,)692-9/$,268,.-$*,&'$G''2$,+0 +(9&'7$G4$)*'$C2-)6)3)692,.$T)*61,.$E9886))''$9/$ `,G9(,)9(4$9/$R)'8$E'..$Q'-',(1*$,27$a++.61,)692$]26&'(-6)4$9/$R16'21'5$b6')2,8$c,)692,.$]26&'(-6)4$ b6')2,8>$$

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Conflict of Interest

F*'$,3)*9(-$*,&'$7'1.,('7$)*,)$)*'('$6-$29$192/.61)$9/$ 62)'('-)>$$

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